

OBSERVATIONS ON THE PROTECTIVE ACTION
OF NORMAL SERUM IN EXPERIMENTAL INFECTION WITH
BACILLUS DIPHThERIAE.

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In carrying out test inoculations in guinea-pigs with *B. diphtheriae* and diphtheroid bacilli for purposes of identification, where control animals were injected with the particular strain plus a certain amount of diphtheria antitoxin, it was noted that these animals were protected against lethal doses of *B. diphtheriae* by normal horse serum as well as by the specific immune serum. Though this observation did not involve any quantitative comparisons between the effect of the normal and the immune serum, the fact that normal horse serum should act protectively in the same way as the serum of a specifically immunised animal seemed of considerable importance, and suggested the further investigation of the phenomenon. This normal-serum effect is also of general interest in relation to "non-specific therapy," where alien protein (*e.g.* animal serum, bacterial protein, etc.), injected parenterally, is found to exert a favourable influence on the course of various infections in a non-specific manner.

With a view, therefore, to analysing the effect, a series of experiments were carried out which fully established the original observation, and yielded further information of some theoretic and practical interest.

The protective and curative action of normal horse serum in *B. diphtheriae* infection and intoxication has also been studied by other workers.

Kolle and Schlossberger concluded that in guinea-pigs infected with *B. diphtheriae* normal horse serum had a limited curative effect, which was in no way comparable with that of an antitoxic serum, and in the case of animals injected with diphtheria toxin had a certain "delaying" action but was not definitely curative. They stated that the effect was "non-specific, resistance-increasing, stimulating," but did not offer any further explanation of their results. Kraus and Sordelli claimed that normal horse serum contains antitoxin to diphtheria toxin, and in virtue of this exerts a "preventive" effect against *B. diphtheriae* and its toxin. Cowie and Greenthal showed that 1 c.c. of normal horse serum injected subcutaneously

and intravenously in guinea-pigs simultaneously with diphtheria toxin always protects against 1 M.L.D., and may neutralise even 8 M.L.D.; they showed that the activity of the serum depends on the proteins and not on the alcohol-soluble fraction. They regarded the protective power as not entirely due to a natural diphtheria antitoxin.

In the writer's original experiments guinea-pigs of approximately 350 grms. weight were injected subcutaneously with 2 c.c. of a 2-days bouillon culture of virulent strains of *B. diphtheriae*, and at the same time 5–10 c.c. of normal horse serum were injected subcutaneously at a different site from that of inoculation; these animals survived, while animals of similar weight, injected with the same amount of the same cultures, died within 24 to 48 hours, showing at autopsy the characteristic effects of *B. diphtheriae* inoculations. Various strains and different specimens of normal horse serum gave the same results.

For the detailed investigation, particular strains of *B. diphtheriae* isolated from typical cases of throat diphtheria were used.

DEGREE OF PROTECTIVE ACTION OF NORMAL HORSE SERUM.

In order to ascertain quantitatively the degree of protection conferred by normal serum, guinea-pigs of approximately equal weight were injected subcutaneously with a fixed quantity of a *B. diphtheriae* bouillon culture, and at the same time with varying amounts of horse serum; the minimum lethal dose of the culture was also estimated in parallel experiment (Table I).

Thus 2 c.c. of serum protected against at least 12 lethal doses of the culture (*i.e.* 1 c.c. serum against 6 M.L.D.).

TABLE I.

Guinea-pigs approximately 400 grms. weight injected subcutaneously in flank with 3 c.c. of a 2-days bouillon culture of *B. diphtheriae*, "strain 3"; varying amounts of normal horse serum injected subcutaneously in back.

1.	<i>B. diphtheriae</i> + 6 c.c.	serum	.	.	survived.
2.	" + 5 c.c.	"	.	.	"
3.	" + 4 c.c.	"	.	.	"
4.	" + 3 c.c.	"	.	.	"
5.	" + 2 c.c.	"	.	.	"
6.	" + 1 c.c.	"	.	.	died 6th day.
7.	" + 0.5 c.c.	"	.	.	" 4th "
8.	" + 0.25 c.c.	"	.	.	" 3rd "
9.	" + 0.1 c.c.	"	.	.	" 4th "
10.	" no serum	"	.	.	" 2nd "

MINIMUM LETHAL DOSE ESTIMATION.

Guinea-pigs approximately 400 grms. weight: same culture injected subcutaneously.

1.	B. diphtheriae bouillon culture,	1.5 c.c.	.	.	died 2nd day.
2.	"	"	"	1.0 c.c.	" 2nd "
3.	"	"	"	0.5 c.c.	" 4th "
4.	"	"	"	0.25 c.c.	" 7th "
5.	"	"	"	0.1 c.c.	survived.
6.	"	"	"	0.05 c.c.	"

PROPHYLACTIC AS OPPOSED TO CURATIVE EFFECT.

It was found that normal serum, though protective or prophylactic, was not curative; thus, to elicit the antagonistic effect, the serum had to be injected without delay after the introduction of the infecting organisms; if an interval of two hours or longer was allowed to elapse before the serum was injected, even when five times the protective dose was used, the animals died (Table II).

THERMOSTABILITY OF THE ACTIVE SUBSTANCE IN THE SERUM.

In thermostability the active principle of the serum corresponds generally to antitoxin and other antibodies. Horse serum heated for one hour at 57° C. was still protective, but was inactivated by exposure at 70° C. for half an hour and at higher temperatures (Table III). The thermostability at 57° C. excluded the possibility of the active substance being of complement nature.

TABLE II.

Guinea-pigs approximately 400 grms. weight injected subcutaneously in flank with 3 c.c. 2-days bouillon culture of *B. diphtheriae*, "strain 3"; 10 c.c. normal horse serum injected subcutaneously in back at varying intervals after injection of the culture.

1.	B. diphtheriae+serum,	simultaneously	.	.	survived.
2.	"	+	"	after 2 hours	died 3rd day.
3.	"	+	"	" 4 "	" 2nd "
4.	"	+	"	" 24 "	" 2nd "
5.	"	no serum.	.	.	" 2nd "

TABLE III.

Guinea-pigs approximately 450 grms. weight injected subcutaneously in flank with 3 c.c. of a 2-days bouillon culture of *B. diphtheriae*,

"strain 2"; normal horse serum injected subcutaneously in back at same time.

1.	B. diphtheriae	+10 c.c. unheated serum	. survived.
2.	"	no serum	. died 2nd day.
3.	"	+10 c.c. serum, 57° C., 1 hr.	. survived.
*4.	"	+50 c.c. of a serum dilution, 1 part in 5 of normal saline, heated at 70° C., $\frac{1}{2}$ hr.	. died 2nd day.
*5.	"	+60 c.c. of a serum dilution, 1 part in 5 of normal saline, heated at 90° C. $\frac{1}{2}$ hr.	. died 2nd day.
*6.	"	+50 c.c. of a serum dilution, 1 part in 5 of normal saline, heated at 100° C. for 5 minutes	. died in 24 hours, showing more marked lesions than other animals; marked haemorrhagic in- flammatory oedema at site of inoculation, with marked haemorrhages in suprarenals, lungs, and intestinal wall.

EXPERIMENTS WITH RABBITS.

Using rabbits as the test animal, horse serum was found to exert the same protective action against *B. diphtheriae* as in the case of guinea-pigs; the particular effect was not therefore limited to *B. diphtheriae* infection in one species of animal only. Table IV exemplifies one of these experiments.

TABLE IV.

Rabbits of equal weight injected subcutaneously with 4 c.c. of a 3-days bouillon culture of *B. diphtheriae*, "strain 3."

1. +20 c.c. normal horse serum injected
subcutaneously at different site . survived.
2. No serum died 3rd day.

* The serum was diluted to prevent coagulation on heating.

PROTECTIVE ACTION OF THE SERUM OF DIFFERENT ANIMALS.

The serum from various animal species (ox, sheep, man, rabbit, cat) was found to confer the same protection as horse serum; in the experiments of other workers on this subject the phenomenon has been studied from the point of view apparently of its being related to horse serum only; the effect is, however, not characteristic of the serum of only one species of animal (Table V).

The question then arose as to whether the serum of one guinea-pig injected parenterally into another guinea-pig would protect the latter against *B. diphtheriae* infection. To ascertain this a number of experiments were carried out, but with variable results: in some cases complete protection was obtained; in others, while the infected animals succumbed to the dose given, there was a distinct delaying of the lethal effect (Table VI). Thus it was apparent that though protection by normal serum from an alien species was the general rule, the serum of the same species was not without a similar effect.

TABLE V.

Guinea-pigs approximately 450 grms. weight injected subcutaneously in flank with 3 c.c. of a 2-days bouillon culture of *B. diphtheriae*, "strain 2"; serum of various animals injected subcutaneously in back at same time.

1.	<i>B. diphtheriae</i> +10 c.c. ox serum	.	.	.	survived.
2.	„ +10 c.c. sheep's serum	.	.	.	„
3.	„ + 8 c.c. human serum	.	.	.	„
4.	„ +10 c.c. cat's serum	.	.	.	„
5.	„ + 6 c.c. rabbit's serum	.	.	.	„
6.	„ + 3 c.c. „ „ (55° C.)	.	.	.	„
7.	„ +10 c.c. horse serum	.	.	.	„
8.	„ no serum.	.	.	.	died 2nd day.

TABLE VI.

Guinea-pigs approximately 400 grms. weight injected subcutaneously with 3 c.c. 2-days bouillon culture of *B. diphtheriae*; serum from other guinea-pigs injected subcutaneously at a different site.

1.	(a) <i>B. diphtheriae</i> , "strain 2"+6 c.c. serum	.	.	.	died 6th day.
	(b) control (no serum injected)	.	.	.	„ 2nd „
2.	<i>B. diphtheriae</i> , "strain 2"+8 c.c. serum (55° C.)	.	.	.	survived.
	(b) control	.	.	.	died 2nd day.

3. (a) *B. diphtheriae*, "strain 2" + 10 c.c. serum (from 2 animals; sera pooled) died 19th day.
 (b) control " 2nd "
4. (a) *B. diphtheriae*, "strain 3" + 5 c.c. serum (from 2 animals), and 5 c.c. following day . . . survived.
 (b) control died 2nd day.
5. (a) *B. diphtheriae*, "strain 3" + 10 c.c. serum . . . survived.
 (b) control died 2nd day.
6. (a) *B. diphtheriae*, "strain 3" + 10 c.c. serum . . . " 5th "
 (b) control " 2nd "
7. (a) *B. diphtheriae*, "strain 3" + 20 c.c. serum . . . " 8th "
 (b) control " 2nd "
8. (a) *B. diphtheriae*, "strain 3" + 10 c.c. serum . . . survived.
 (b) control died 3rd day.

It is a phenomenon of considerable interest that the serum of one individual of an animal species, which is highly susceptible to experimental *B. diphtheriae* infection, should be capable of affording some protection, when injected parenterally in certain amounts, to another individual of the same species infected with the particular organism.

EXPERIMENTS WITH OTHER PROTEINS ;

BACTERICIDAL EXPERIMENTS.

Experiments were carried out with egg-albumen, milk, and peptone to determine whether the parenteral injection of these proteins would confer any protection as in the case of the serum proteins. The results were uniformly negative.

In vitro bactericidal tests were also made to ascertain whether horse serum (in varying amounts), along with fresh guinea-pig's serum as complement, exerted a specific bactericidal action on *B. diphtheriae*, but no such effect could be demonstrated, thus excluding the possibility of the protection being due to a normal bactericidal immune body in horse serum.

EXPERIMENTS WITH DIPHTHERIA TOXIN.

Experiments were then carried out to ascertain whether the protective effect was due to a natural antitoxic action on the part of the serum injected, though it seemed difficult to explain on this basis the action of guinea-pig's serum in the case of infection in the same species.

A powerful toxin was prepared from *B. diphtheriae*, "strain 3." It was found that 10 c.c. of horse serum was protective against 10 M.L.D. of the

toxin (1 c.c. against 1 M.L.D.), whereas 1.5 c.c. of diphtheria antitoxin, representing 1000 immunity units, protected against 10,000 M.L.D. The minimum lethal dose was estimated in parallel experiments along with the protective tests (Table VII).

A similar series of experiments were carried out in which the pooled serum of three guinea-pigs was substituted for horse serum (Table VIII).

The parenteral injection of 10 c.c. of guinea-pig's serum had apparently no protective action in guinea-pigs against B. diphtheria toxin.

TABLE VII.

Guinea-pigs 250 grms. weight injected subcutaneously in flank with varying doses of diphtheria toxin; at the same time injected subcutaneously in back with 10 c.c. normal horse serum.

1.	Toxin 0.1	c.c.+10 c.c. serum	.	.	died 2nd day.
2.	„ 0.05	c.c.+ „	.	.	„ 2nd „
3.	„ 0.01	c.c.+ „	.	.	„ 7th „
4.	„ 0.005	c.c.+ „	.	.	„ 12th „
5.	„ 0.001	c.c.+ „	.	.	survived.
6.	„ 0.0005	c.c.+ „	.	.	„
7.	„ 1	c.c.+1000 immunity units			
		diphtheria antitoxin			„

M.L.D. Estimation.

1.	Toxin 0.1	c.c.	.	.	died 2nd day.
2.	„ 0.01	c.c.	.	.	„ 2nd „
3.	„ 0.001	c.c.	.	.	„ 2nd „
4.	„ 0.0005	c.c.	.	.	„ 2nd „
5.	„ 0.0001	c.c.	.	.	„ 3rd „
6.	„ 0.00005	c.c.	.	.	survived.
7.	„ 0.00001	c.c.	.	.	„

M.L.D.—0.0001 c.c.

NOTE.—The small doses of toxin 0.05 c.c.—0.00001 c.c. were measured by preparing decimal dilutions of the toxin 1 : 10, 1 : 100, 1 : 1000, 1 : 10,000, 1 : 100,000 and injecting the appropriate quantities of these dilutions.

TABLE VIII.

Guinea-pigs 250 grms. weight injected subcutaneously in flank with varying doses of diphtheria toxin; at the same time 10 c.c. of guinea-pig's serum (from three animals, pooled) injected subcutaneously in back.

1.	Toxin 0-001	c.c.+10 c.c. serum	.	.	died 2nd day
2.	„ 0-0001	c.c.+ „	.	.	„ 2nd „
3.	„ 0-00005	c.c.+ „	.	.	survived.
4.	„ 0-00001	c.c.+ „	.	.	„

Lethal Dose Estimation.

1.	Toxin 0-001	c.c. . .	.	.	died 2nd day.
2.	„ 0-0001	c.c. . .	.	.	„ 2nd „
3.	„ 0-00005	c.c. . .	.	.	survived.
4.	„ 0-00001	c.c. . .	.	.	„

THE OCCURRENCE OF LOCAL LESIONS IN ANIMALS SURVIVING AFTER
PROTECTION BY SERUM.

An interesting feature of these experiments was the occurrence of a marked local lesion in animals surviving after protection by normal serum against *B. diphtheriae* or its toxin.

After two to three days a well-marked subcutaneous indurated swelling with surrounding inflammatory oedema developed at the site of injection of the organisms or toxin; the overlying skin then became necrotic and sloughed, leaving a punched-out ulcer with a grey sloughing base; the lesion attained its full development in eight to ten days, and then slowly healed; in one instance the lesion involved the deeper tissues, and led to perforation of the whole abdominal wall.

It was found that diphtheria antitoxin prevented the occurrence of the local lesion as well as the general effects.

This type of lesion occurred in rabbits protected by serum as well as in the case of guinea-pigs.

In the infection experiments no diphtheria bacilli could be detected in the ulcers after sloughing of the skin, either by microscopic examination or culture. On the other hand, in experiments where the injection of guinea-pig serum delayed the lethal effect of *B. diphtheriae* for several days (*e.g.* eight days, nineteen days, *v.* Table VI), and similar sloughing ulcers developed at the site of inoculation, *B. diphtheriae* were present in the lesion.

Thus the parenteral injection of normal serum, though protecting against the lethal result of infection with *B. diphtheriae*, had no influence on its local toxic action. It was apparent also that *B. diphtheriae* did not persist in the tissues of animals fully protected by serum.

In unprotected animals the local lesion of the type described is not commonly met with, as animals infected with a sufficient dose die within a few days; for the full development of the local effect a period of eight to

ten days is required, and it was apparent that the occurrence of this particular lesion in protected animals was simply dependent on the survival of the animal. In rabbits, which are less susceptible to experimental *B. diphtheriae* infection and resist larger doses than guinea-pigs, it has been noted that sub-lethal doses may produce similar marked local lesions apart from any serum protection.

DISCUSSION.

These observations represent a further contribution to the study of non-specific immunisation by parenteral injection of normal serum.

While it is possible that in the case of horse serum a natural antitoxin may contribute to the protection noted, and it is of importance that the active substance in the serum corresponds to antibodies in thermostability, the action of the serum in bringing about some other antagonistic effect must be considered. This effect apparently does not depend on the transference of bactericidal complement or a natural bactericidal antibody; the serum is protective against infection apart from intoxication; thus the organisms are absent from the local lesion. It might be claimed, however, that the antitoxic factor contributes to the elimination of the bacteria by rendering them more susceptible to phagocytosis through neutralisation of their toxin. It is to be noted also that normal serum is not antitoxic as regards the local toxic effects, whereas diphtheria antitoxin protects animals against local as well as general effects. The fact that the parenteral injection of the serum of one guinea-pig into another exerts a "delaying" effect in diphtheria infections and even complete protection, though this species has little natural immunity to *B. diphtheriae* and no natural antitoxin can be demonstrated in the serum, clearly indicates that some mechanism other than that of a natural antitoxin or other antistubstance transferred with the serum plays the important part in increasing the resistance of the animal.

These experiments are therefore of interest in their bearing on the general question of "non-specific immunity," and provide a further illustration of how the resistance of the tissues to a particular organism may be enhanced by the parenteral injection of normal serum. The results, however, clearly exclude the possibility that normal horse serum could be as effective a therapeutic agent as a potent diphtheria antitoxin.

CONCLUSIONS.

1. In guinea-pigs experimentally infected with *B. diphtheriae*, normal serum (horse, ox, sheep, cat, human), injected subcutaneously at the same time as the inoculation, exerts a definite protective action.

2. Two c.c. of normal horse serum may protect in this way against 12 M.L.D. of a *B. diphtheriae* culture.

3. No protection occurs if the serum injection is delayed for two hours after the inoculation.

4. The activity of the serum persists at 57° C., but is lost at 70° C. and higher temperatures.

5. The serum of one guinea-pig injected subcutaneously into another is either protective to the latter experimentally infected with *B. diphtheriae*, or at least exerts a definite delaying effect on the course of the infection.

6. Normal horse serum is also similarly protective in guinea-pigs injected with diphtheria toxin; 10 c.c. of serum may protect against 10 M.L.D.

7. The serum of one guinea-pig is not protective in the case of another injected with diphtheria toxin.

8. In the case of animals surviving after protection by normal serum, a marked local lesion develops at the site of inoculation.

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